The *CesA* Gene Family of Barley. Quantitative Analysis of Transcripts Reveals Two Groups of Co-Expressed Genes[1]

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Sequence data from cDNA and genomic clones, coupled with analyses of expressed sequence tag databases, indicate that the *CesA* (cellulose synthase) gene family from barley (*Hordeum vulgare*) has at least eight members, which are distributed across the genome. Quantitative polymerase chain reaction has been used to determine the relative abundance of mRNA transcripts for individual *HvCesA* genes in vegetative and floral tissues, at different stages of development. To ensure accurate expression profiling, geometric averaging of multiple internal control gene transcripts has been applied for the normalization of transcript abundance. Total *HvCesA* mRNA levels are highest in coleoptiles, roots, and stems and much lower in floral tissues, early developing grain, and in the elongation zone of leaves. In most tissues, *HvCesA1*, *HvCesA2*, and *HvCesA6* predominate, and their relative abundance is very similar; these genes appear to be coordinately transcribed. A second group, comprising *HvCesA4*, *HvCesA7*, and *HvCesA8*, also appears to be coordinately transcribed, most obviously in maturing stem and root tissues. The *HvCesA3* expression pattern does not fall into either of these two groups, and *HvCesA5* transcript levels are extremely low in all tissues. Thus, the *HvCesA* genes fall into two general groups of three genes with respect to mRNA abundance, and the co-expression of the groups identifies their products as candidates for the rosettes that are involved in cellulose biosynthesis at the plasma membrane. Phylogenetic analysis allows the two groups of genes to be linked with orthologous Arabidopsis *CesA* genes that have been implicated in primary and secondary wall synthesis.

Cellulose biosynthesis in vascular plants is effected at the plasma membrane by a rosette terminal complex of proteins that contains catalytic cellulose synthase subunits (Roelofsen, 1958; Mueller and Brown, 1980; Kimura et al., 1999) and, in all likelihood, ancillary proteins or enzymes required for the extrusion of cellulosic chains and assembly of microfibrils (Doblin et al., 2002). In the single most convincing demonstration of high-level in vitro cellulose biosynthesis by plant enzymes, the rosette complexes can been seen at the termini of cellulose microfibrils synthesized in vitro by membrane extracts of suspension-cultured cells of *Rubus fruticosus* (Lai-Kee-Him et al., 2002). Although biochemical approaches to the purification and characterization of plant cellulose synthases have met with little success (Delmer, 1999), mutational genetics, gene silencing, and herbicide studies are now providing overwhelming evidence that the catalytic subunits of rosettes are encoded by *CesA* (cellulose synthase) genes (Pear et al., 1996; Arioli et al., 1998; Burton et al., 2000; Scheible et al., 2001).

Genome sequencing programs and the generation of extensive expressed sequence tag (EST) databases have shown further that plant *CesA* genes are members of multigene families. There are at least 10 *CesA* genes in Arabidopsis, 12 in rice (*Oryza sativa*; Richmond and Somerville, 2000; http://cellwall.stanford.edu/), and at least nine in maize (*Zea mays*; Holland et al., 2000; Dhugga, 2001). In Arabidopsis, mutations in individual *CesA* genes have been linked with cellulose deficiencies in various tissues (Arioli et al., 1998; Taylor et al., 1999, 2000, 2003; Fagard et al., 2000; Scheible et al., 2001; Beeckman et al., 2002; Burn et al., 2002; Caño-Delgado et al., 2003; Gardiner et al., 2003) and with resistance to herbicides that target cellulose biosynthesis (Scheible et al., 2001; Desprez et al., 2002). The individual *CesA* genes of Arabidopsis appear to have evolved specialized functions, which require different genes for expression in different tissues, in primary or secondary wall synthesis, or as multiple components of the cellulose-synthesizing rosettes. In the last case, it has been suggested that several distinct CesA proteins might be necessary for the correct assembly of rosettes in Arabidopsis (Doblin et al., 2002; Taylor et al., 2003).

Additional specialized roles for members of the *CesA* gene family might include the synthesis of wall polysaccharides other than cellulose. Given that the backbone structures of non-cellulosic wall components such as heteroxylans, xyloglucans, mannans, and (1→3,1→4)-β-D-glucans are chemically analogous with cellulose (Fincher and Stone, 1993; Carpita, 1996), it is reasonable to predict that genes required for their synthesis could reside in the *CesA* gene family or in the *Csl* (cellulose synthase-like) gene family (Dhugga, 2001; Vergara and Carpita, 2001; Doblin et al., 2002).

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Here, the *CesA* gene family from barley (*Hordeum vulgare*) has been examined, through cloned cDNAs, EST sequence analysis, and genomic clones, and the locations of the genes on high-density genetic maps have been defined. Quantitative-PCR (Q-PCR; Fink et al., 1998), normalized through multiple internal control genes (Vandesompele et al., 2002), has been used to monitor the abundance of individual *HvCesA* mRNAs in various tissues, with a view to comparing transcript abundance with known differences in cell wall composition in different tissues and at different stages of development. Transcript profiles of members of the barley *CesA* gene family are markedly different from those of maize (Holland et al., 2000; Dhugga, 2001). Co-expression of two groups of genes, namely *HvCesA1*, *HvCesA2*, and *HvCesA6* in one group and *HvCesA4*, *HvCesA7*, and *HvCesA8* in the other, is consistent with the participation of three CesA subunits in rosettes during cellulose synthesis and with the participation of distinct groups of *CesA* genes in primary and secondary wall assembly.

RESULTS

Cloning the *HvCesA* cDNAs and Genes

A PCR product was initially amplified from a young barley leaf cDNA preparation with degenerate primers from conserved regions of plant *CesA* genes. This generated a cDNA, designated *HvCesA1*, which was used to screen a barley suspension-cultured cell cDNA library at low stringency, to yield corresponding fragments of the *HvCesA2* and *HvCesA3* genes. The *HvCesA4* cDNA was first isolated from a 3-d coleoptile library during EST sequencing carried out by Dr. Andreas Graner (Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany). Contiguous sequences for *HvCesA5* and *HvCesA6* were initially constructed from ESTs listed on the http://cellwall.stanford.edu/Web site, and *HvCesA8* was constructed by bridging two singletons listed on the same Web site. The sequences of *HvCesA1*, *HvCesA2*, and *HvCesA3* were extended through EST sequences from the web site. A 3′-untranslated region (UTR) of *HvCesA7*, which is 96% identical with *HvCesA5*, was obtained during Q-PCR experiments. Barley bacterial artificial chromosome (BAC) clones corresponding to most of the individual *HvCesA*s were also identified as follows: *HvCesA1*, 337H09; *HvCesA2*, 69M22; *HvCesA3*, 283N14; *HvCesA4*, 45J23; *HvCesA5/7*, 627G13; and *HvCesA6*, 453O07. Sequences from the BAC clones were used to extend 5′ sequences of the cDNAs. Once the sequences were assembled, PCR was used to generate near full-length (3.6–3.9 kb) single cDNA fragments corresponding to each of the *HvCesA1*, *HvCesA2*, and *HvCesA6* genes. The other cDNAs were truncated at their 5′ ends by between 30 bp and about 1.8 kb because the corresponding BAC clones did not contain the 5′ regions of the genes.

The respective sizes of cDNAs for *HvCesA1*, *HvCesA2*, *HvCesA3*, *HvCesA4*, *HvCesA5/7*, *HvCesA6*, and *HvCesA8* were 3,614, 3,910, 3,180, 1,814, 2,769, 3,739, and 1,246 bp. All have open reading frames that encode polypeptides of 1,000 to 1,100 amino acid residues. Their sequences have been submitted to the databases under accession numbers AY483150, AY483152, AY483151, AY483154, AY483153, AY483155, and AY483156, respectively.

EST Analyses

The sequences of the barley cDNAs are reconciled with available EST sequences in Figure 1, where most ESTs in the public databases can be assigned to the genes cloned here. On this basis, it is concluded that the barley *CesA* gene family has at least eight members. Several singleton EST sequences are currently unassigned, and, although sequence data for ESTs are not always accurate, it is possible that these could represent additional *HvCesA* genes.

Map Locations of the Barley *HvCesA* Genes

The *HvCesA* genes were mapped using several mapping populations and their positions in relation to DNA molecular markers are shown in Figure 2. The precise map positions of the barley genes have been lodged on the GrainGenes Web page under the *CesA* designation (http://wheat.pw.usda.gov/index.shtml). No polymorphisms for the partial *HvCesA4* cDNA were detected in any of the parental lines, so this gene could not be mapped to a specific locus, and the *HvCesA8* gene has not yet been mapped. However, the *HvCesA4* gene has been mapped to the long arm of chromosome 1H, using wheat barley addition lines (Islam et al., 1981; data not shown). The other members of the *HvCesA* gene family are scattered across the genome on chromosomes 2H, 3H, 5H, and 6H (Fig. 2). Two polymorphisms were detected and mapped using the *HvCesA5* probe, and although this is now attributable to close sequence similarity of the *HvCesA5* and *HvCesA7* genes, it is not possible to distinguish between the *HvCesA5* and *HvCesA7* map positions. No phenotypic or barley quality quantitative trait loci obviously associated with cellulose biosynthesis were present in the vicinity of the *HvCesA* genes (data not shown).

Phylogenetic Relatedness of the Barley Genes with other Plant *CesA* Genes

An unrooted, radial phylogenetic tree of the barley *HvCesA* gene family was generated with the software program ClustalX (Thompson et al., 1997; Fig. 3), using amino acid sequences deduced from cDNA sequences that are currently available. A clustering pattern similar to that seen in Figure 3 was also observed if the phylogenetic tree was generated us-

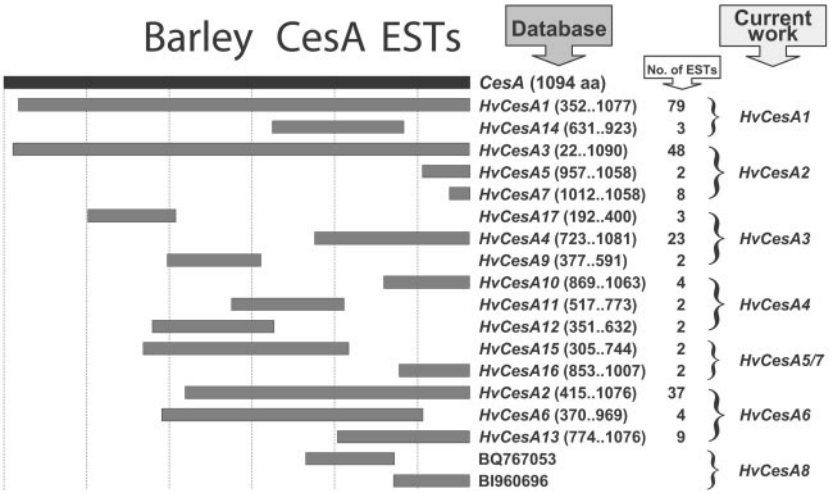

Figure 1. Analysis of EST sequences and the barley *CesA* gene family. More than 314,000 barley ESTs were searched for *CesA* sequences and reconciled against the *HvCesA* genes and cDNAs cloned here (current work) and the *HvCesA* genes listed on the Stanford Web page (http://cellwall.stanford.edu/). The database EST sequences, with the amino acid positions in parentheses, are aligned with the cDNA sequences. The numbers of ESTs recorded in the databases for each cDNA fragment are indicated in the central column. The relatively long cDNAs cloned in the present work enabled the putative number of expressed barley *HvCesA* genes in the databases to be reduced from 19 to about eight.

ing only the hypervariable regions of the genes (Vergara and Carpita, 2001; data not shown). As observed in the EST analysis, several pairs of *CesA* genes from maize, rice, and barley were very closely related, including *ZmCesA1/ZmCesA2*, *ZmCesA4/ZmCesA9*, *OsCesA6/OsCesA11*, and *HvCesA5/HvCesA7*. Amino acid sequence identities between the Arabidopsis and barley CesA proteins are shown in Table I.

Normalization Factors (NFs) for Q-PCR

The relative levels of individual *HvCesA* mRNAs were determined using real-time Q-PCR (Fink et al., 1998). To avoid confusion with reverse transcriptase (RT)-PCR, we have adopted here the term Q-PCR in preference to "real-time" PCR. A key requirement for the accuracy of expression profiling by Q-PCR is the development of a rigorous normalization strategy (Vandesompele et al., 2002). In the experiments described here, mRNA levels for a series of four stably expressed genes, including glyceraldehyde-3-phosphate dehydrogenase (*HvGAPDH*), α-tubulin (*HvTub*), heat shock protein 70 (*HvHSP70*), and cyclophilin (*HvCycl*), were evaluated as control genes for the Q-PCR analyses and for the calculation of reliable NFs used in comparisons between cDNAs from different tissues. For each control gene, gene-specific primers were used and individual genes were selected on the basis of their high-level, stable transcriptional activity in a range of barley tissues (our unpublished data; Haendler et al., 1987; Bustin,

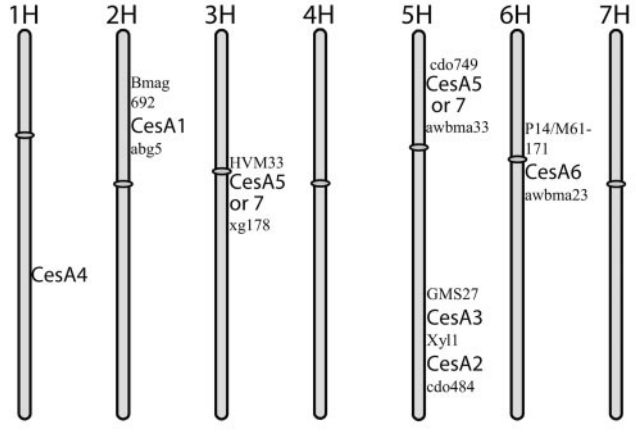

Figure 2. Map locations for the *HvCesA* genes. Flanking DNA markers for each of the genes are indicated. No polymorphisms were detected for *HvCesA4*, which could only be mapped to the long arm of chromosome 1H. Two map positions were determined using the *HvCesA5/A7* probe, but it was not possible to define which corresponded to the *HvCesA5* and *HvCesA7* genes. The exact positions of the genes are available on the GrainGenes Web page (http://wheat.pw.usda.gov/index.html).

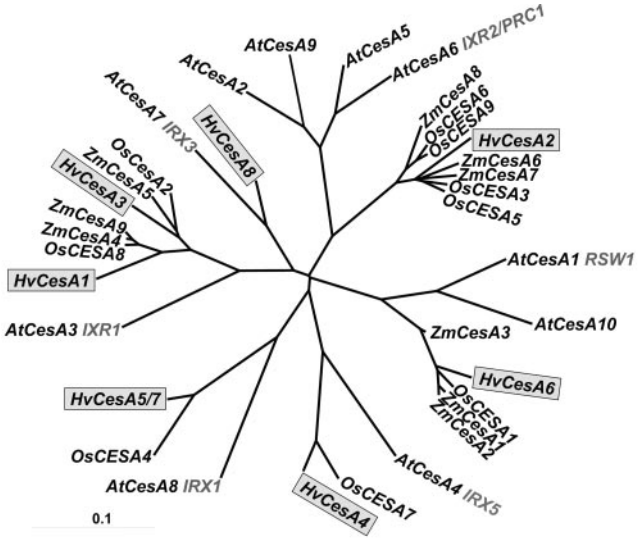

Figure 3. Phylogenetic relatedness of the barley cellulose synthase (*HvCesA*) genes and those from Arabidopsis (*AtCesA*), maize (*ZmCesA*), and rice (*OsCesA*). The phylogenetic tree was generated with the ClustalX program and is based on amino acid sequence identities. Where the function of the gene has been implicated in a function relating to cellulose synthesis in Arabidopsis, the locus name is indicated (e.g. *PRC1*, *IRX*, *IXR*, and *RSW*).

Table I. *Amino acid sequence identities of barley (Hv) and Arabidopsis (At) CesA proteins*

Identities are expressed as percentages, along the complete open reading frames of the encoded proteins. Underlined values indicate orthologous Arabidopsis and barley sequences.

Gene	Barley HvCesA Sequences							Arabidopsis AtCesA Sequences									
	Hv1	Hv2	Hv3	Hv4	Hv5/7	Hv6	Hv8	At1	At2	At3	At4	At5	At6	At7	At8	At9	At10
								%									
HvCesA1	100	–	–	–	–	–	–	66	61	76	62	61	60	66	57	60	63
HvCesA2	63	100	–	–	–	–	–	66	69	66	63	70	69	66	58	69	63
HvCesA3	83	65	100	–	–	–	–	69	63	77	63	63	61	67	58	62	67
HvCesA4	69	69	69	100	–	–	–	71	67	70	79	67	67	71	68	66	68
HvCesA5/7	67	65	68	70	100	–	–	69	65	69	66	65	64	68	73	65	67
HvCesA6	67	64	69	69	69	100	–	79	64	68	63	64	63	64	62	63	76
HvCesA8	78	76	81	76	72	77	100	78	72	82	77	71	70	89	71	71	75

2000; Ozturk et al., 2002). One of the cellulose synthase (*HvCesA1*) transcripts also proved to be useful in normalization.

The NF, derived from the geometric mean of expression data for the most stably expressed control genes by the geNorm program (Vandesompele et al., 2002), was calculated from the five control genes. Average expression stability for individual genes is calculated as an M value; a low M value is indicative of more stable expression and, hence, increased suitability of a particular gene as a control gene (Vandesompele et al., 2002). In a processive calculation, the gene with the highest M value is eliminated and the NF for the remaining genes recalculated until M values are essentially constant.

In the present work, NF values for most cDNA samples remained relatively unchanged between five and two control genes, except that the NFs for the root tip sample decreased significantly between the use of five and two control genes (Fig. 4). The order of elimination of control genes was *HvCesA1*, *HvHSP70*, and *HvTub*, leaving *HvCycl* and *HvGAPDH*. It can be seen in Figure 4 that the elimination of *HvTub* to leave just two control genes significantly decreased the NF value for the root tip cDNA. The addition of *HvHSP70* markedly increased the root tip NF value and at the same time introduced a gene with expression that varies more widely across the tissue series, that is, it has a higher M value than the best three control genes. On this basis, the NF values based on the control genes *HvTub*, *HvCycl*, and *HvGAPDH* were used for normalization of the expression data of the *HvCesA* gene family (Table II). Normalized expression data from the *HvCesA* genes were calculated by dividing the raw data by the NF value. SDs were calculated from four replicate PCR experiments per cDNA sample.

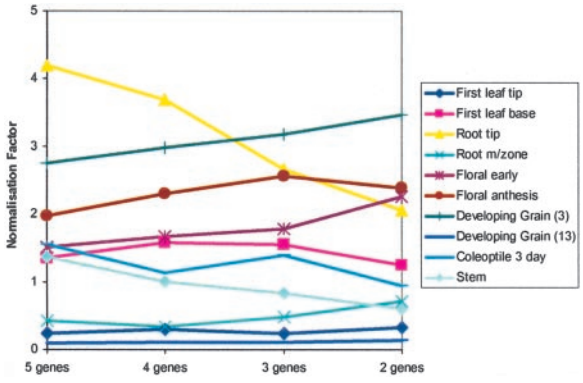

Figure 4. Q-PCR NFs calculated for the cDNAs of the 10 tissues, based on the five control genes and recalculated after the sequential removal of the most variable control gene, that is the gene with the highest M value. Before the cDNA synthesis, total RNA was isolated from the ten tissues under the conditions described in "Materials and Methods." Tissues examined include the tip and base of the first leaf (leaf tip and leaf base), the root tip and the maturation zone of roots (root m/zone), flowers just before anthesis (floral early), flowers at anthesis (floral anthesis), developing grain 3 and 13 DPA (developing grain 3 and developing grain 13), coleoptiles (3 d), and the stem of mature plants just below the emerging ear (stem).

Data from the tissue series were used to determine the most appropriate genes for the tissue subseries comprising seedling root, seedling leaf, coleoptile, and mature leaf. When the data were considered for the pair of tissues from leaf or root, the order of fitness of control genes was different from that calculated from all the tissues in the series. This is not surprising because the tissues are metabolically quite distinct (Vandesompele et al., 2002). The best of the barley control genes for the various tissue cDNAs are listed in Table III, together with the NF values that were subsequently used to calculate normalized expression data and errors for the tissue subseries.

Table II. *NFs calculated by geNorm for the tissue series of cDNAs based on the combination of three control genes (HvGAPDH, HvCycl, and HvTub)*

Tissue	NF
First leaf tip	0.24
First leaf base	1.55
Root tip	2.66
Root m/zone	0.49
Floral early	1.79
Floral anthesis	2.57
Developing grain (3)	3.18
Developing grain (13)	0.12
Coleoptile 3 d	1.40
Stem	0.84

Table III. *NFs calculated by geNorm for the tissue subseries of cDNAs based on the combination of the three best control genes*

Tissue	Normalization Genes	NF
Leaf E (base)	Cyclophilin, GAPDH, HSP70	3.15
Leaf D	Cyclophilin, GAPDH, HSP70	0.92
Leaf C	Cyclophilin, GAPDH, HSP70	0.72
Leaf B	Cyclophilin, GAPDH, HSP70	0.54
Leaf A (tip)	Cyclophilin, GAPDH, HSP70	0.89
Mature leaf base	a tubulin GAPDH, *HvCesA1*	0.42
Mature leaf B	a tubulin GAPDH, *HvCesA1*	0.66
Mature leaf C	a tubulin GAPDH, *HvCesA1*	5.29
Mature leaf D	a tubulin GAPDH, *HvCesA1*	0.68
Root 4-m/zone	Cyclophilin, *HvCesA1*, α-tubulin	0.32
Root 3	Cyclophilin, *HvCesA1*, α-tubulin	0.91
Root 2	Cyclophilin, *HvCesA1*, α-tubulin	0.76
Root 1-tip	Cyclophilin, *HvCesA1*, α-tubulin	4.56
Coleoptile base	Cyclophilin, α-tubulin GAPDH	1.25
Coleoptile middle	Cyclophilin, α-tubulin GAPDH	0.36
Coleoptile top	Cyclophilin, α-tubulin GAP	2.19

Transcript Profiling of mRNAs for *HvCesA* Genes in Different Tissues

Overall, *HvCesA* transcript levels were highest in 3-d coleoptiles, stems taken from the fully extended internode below the spike before anthesis, and the maturation zone of roots (Fig. 5A). Levels were relatively low in developing grain and floral tissues. Closer scrutiny of the data in Figure 5A revealed two distinct groups of apparently co-expressed genes. Group I consisted of *HvCesA1*, *HvCesA2*, and *HvCesA6*. Using the primer sets shown in Table IV, *HvCesA1* levels in the majority of tissues were about the same as *HvCesA2*, and both were consistently 2- to 3-fold higher than *HvCesA6* (Fig. 5B). The second group of apparently cotranscribed genes (Group II) consisted of *HvCesA4*, *HvCesA7*, and *HvCesA8* (Fig. 5C). Within Group II, *HvCesA8* transcript levels are consistently about 2-fold higher that *HvCesA4* mRNA, whereas *HvCesA7* transcript levels are about 10% to 15% of *HvCesA8* levels (Fig. 5C). Transcripts of *HvCesA5* were of lowest abundance in all tissues, at 0.001% to 0.09% of *HvCesA1* transcript levels (Fig. 5A), and were detected mainly in mature stem and in the maturation zone of roots. Transcripts of *HvCesA3* were generally low, except in leaf tips (Fig. 5A), and expression patterns of this gene appeared to be independent of the two co-expressed groups.

Abundance of *HvCesA* Transcripts in Segments of Vegetative Tissues

The abundance of individual *HvCesA* mRNAs in various growth zones of vegetative tissues was examined in segments of young seedling leaves (7 d old), young roots (5 d old) and coleoptiles (5 d old), as indicated in Figure 6, and in mature leaves (fifth leaf). In young seedling leaves, levels of most mRNAs were highest in the cell elongation zone above the leaf base (Figs. 7, A and B). Again, levels of the Group I mRNAs of *HvCesA1*, *HvCesA2* and *HvCesA6* were highest and in approximately constant proportions along the seedling leaf (Fig. 7A). Group II mRNAs (*HvCesA4*, *HvCesA7*, and *HvCesA8*) were consistently lower in abundance than the Group I mRNAs but also peaked in the seedling leaf elongation zone (Fig. 7B). However, *HvCesA3* levels showed a completely different distribution pattern, reaching maximal levels in region B, just below the leaf tip (Fig. 7C).

After the observation that *HvCesA3* mRNA levels were relatively abundant in young leaf tips (Figs. 5A and 7C), transcript profiles were examined in segments of mature leaves (Fig. 8). In mature leaves, *HvCesA3* mRNAs were more abundant than those of any other *HvCesA* and peaked in the middle region of the leaf (Fig. 8).

In root segments, two distinct distribution patterns can be seen (Figs. 9, A and B). The Group I *HvCesA1*, *HvCesA2*, and *HvCesA6* transcripts were relatively evenly distributed along the root (Fig. 9A), whereas the Group II *HvCesA4*, *HvCesA7*, and *HvCesA8* transcripts decreased in abundance toward the root tip (Fig. 9B). In these tissue segments, *HvCesA3* transcript levels followed the Group II pattern (data not shown).

In coleoptiles, Group I and II transcripts appear to decrease from the base to the tip (Fig. 10, A and B), although the levels of the Group I transcripts (Fig. 10A) are much higher than the Group II transcripts (Fig. 10B). In the coleoptile sections, *HvCesA3* transcript levels remained relatively high in all segments (data not shown), in contrast to both Groups I and II, which decreased from the base to the top of the coleoptiles (Fig. 10).

DISCUSSION

Near full-length cDNAs for *HvCesA1*, *HvCesA2*, and *HvCesA6* were obtained, together with truncated cDNAs encoding several other *HvCesA* genes. It is concluded at this stage that there are at least eight *CesA* genes in barley and that these are transcribed at levels sufficient to detect through cDNA or EST library screening (Fig. 1). In Arabidopsis, there are 10 *CesA* genes, whereas in rice and maize, there are 12 and at least nine, respectively (Holland et al., 2000; Richmond and Somerville, 2000; Dhugga, 2001). The number of barley BAC clones screened in the present work was calculated to cover 90% of the barley genome. Thus, it is possible that additional *HvCesA* genes are present and that transcripts will be detected in specific tissues not examined here or will have sequences so similar to others that it will not be possible to differentiate between true genetic differences, varietal differences, and sequencing errors. All of the *HvCesA* genes that covered the putative catalytic region of the barley enzymes encode proteins with a D,D,D,QVRRW motif; this is characteristic of

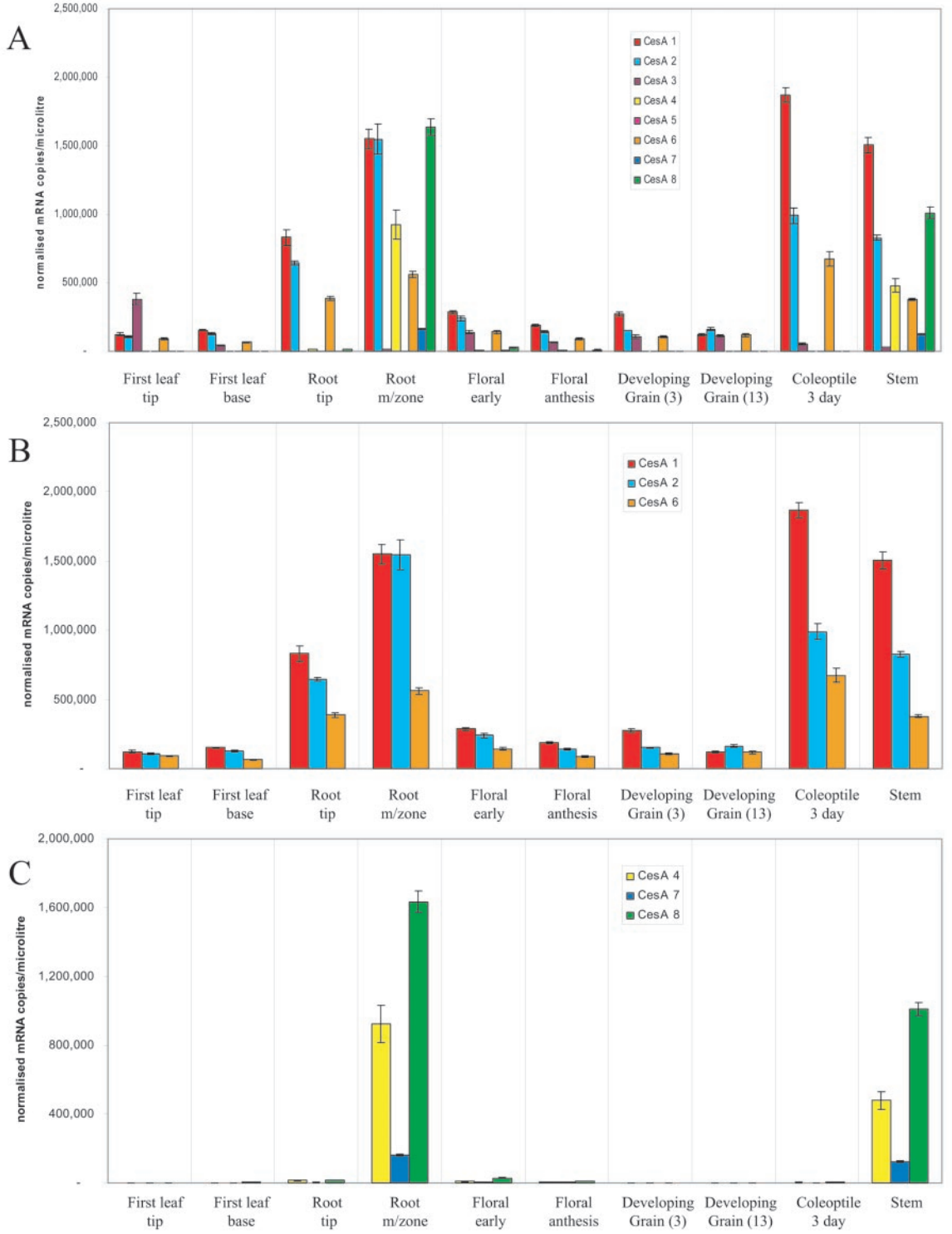

Figure 5. Normalized expression levels of the eight *HvCesA* genes in a range of tissues. Levels of mRNA are presented as number of copies per microliter after normalization. Tissues and their abbreviations are as detailed in the legend to Figure 4. Error bars = SDs for each mRNA. A, Levels of the eight *HvCesA* mRNAs in the 10 different tissues. Very low levels of *HvCesA5* mRNA were detected in some tissues, but the amount could not be seen on the scale used here. B, Levels of *HvCesA1*, *HvCesA2*, and *HvCesA6* transcripts in the different tissues, showing the similar relative abundance of this group of mRNAs (designated Group I). C, Levels of *HvCesA4*, *HvCesA7*, and *HvCesA8* transcripts in the series of tissues, showing the similar relative abundance of this group of transcripts (designated Group II).

Table IV. *PCR primers and PCR product sizes in base pairs together with optimal acquisition temperatures for the genes analyzed*

Gene	Forward Primer	Reverse Primer	PCR Size	Acquisition Temperature
			bp	*°C*
GAPDH	GTGAGGCTGGTGCTGATTACG	TGGTGCAGCTAGCATTTGAGAC	198	80
HSP 70	CGACCAGGGCAACCGCACCAC	ACGGTGTTGATGGGGTTCATG	108	83
α Tubulin	AGTGTCCTGTCCACCCACTC	AGCATGAAGTGGATCCTTGG	248	80
Cyclophilin	CCTGTCGTGTCGTCGGTCTAAA	ACGCAGATCCAGCAGCCTAAAG	122	79
HvCesA 1	TGTGGCATCAACTGCTAGGAAA	CGTACAAAGTGCCTCATAGGAAA	267	75
HvCesA 2	CAGCCAGCCAGCAATTCTTTAT	AACCGCATTCTTGCCTTACAGA	188	75
HvCesA 3	ACACGAGTCACTGGGCCAGA	CTGGTAAACTAGTCACCCGCTGA	122	75
HvCesA 4	GCCCAAGGGACCCATTCTTA	TTACAACTTGGAACCCCCCA	170	76
HvCesA 5	TGAGCAGCTGTCATTGCTTGG	AATAGTAGCCTACATCACCTCCG	141	77
HvCesA 6	GCCGCTGAGAACCACTGTCTAC	GATATACTCTAAGCAACAAAGAACAGGTA	107	75
HvCesA 7	TGAGCAGCTGCCGTTGCTTGG	AATAGTAGCCTACATCACCTCTG	141	77
HVCesA 8	ACAGTTTGGACGCAAGTTTTGTATT	CGGTCCTCTGTTCAATTCTTGTTTA	103	80

plant cellulose synthases (Pear et al., 1996). Molecular mapping studies showed that the *HvCesA* genes are scattered across the barley genome (Fig. 2), as observed for the Arabidopsis and maize *CesA* genes (Holland et al., 2000).

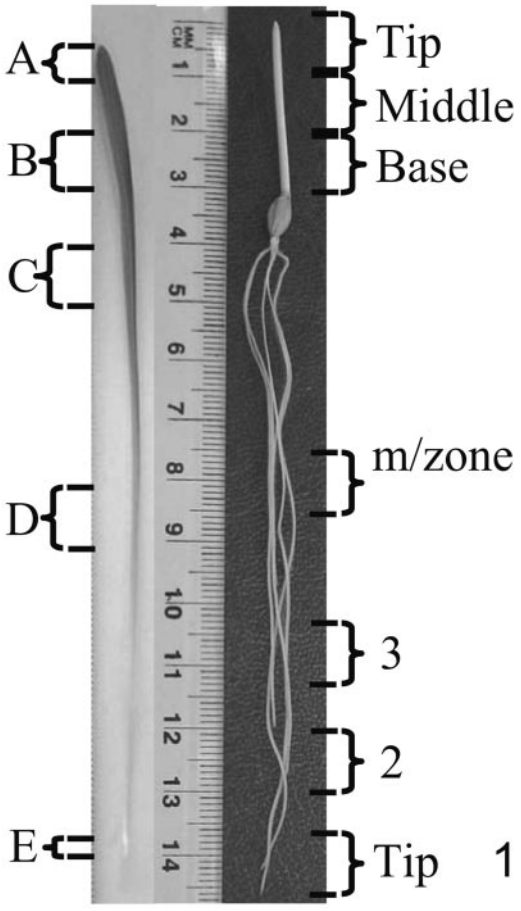

Figure 6. Segments of vegetative tissues used for the isolation of RNA for Q-PCR analysis of transcript abundance. The position of segments A to E of the young first leaf (7 d old) are indicated on the left, and the segments of the 5-d-old coleoptile (tip, middle, and base) and young root (maturation zone, 3, 2, and tip) are shown on the right.

Q-PCR, in which geometric averaging of multiple internal control gene transcripts is important for the normalization of transcript abundance (Vandesompele et al., 2002), was used to quantitate mRNA levels for individual *HvCesA* genes in a range of tissues (Figs. 5 and 7–10). In most tissues and in segments of growing coleoptiles, roots, and leaves, mRNAs for *HvCesA1*, *HvCesA2*, and *HvCesA6*, in that order, were most abundant, and in approximately similar relative concentrations. These co-expression patterns suggested that the corresponding genes might be coordinately transcribed in the tissues examined here (Fig. 5). In accordance, we have designated these three genes as Group I members of the *HvCesA* gene family. Additionally, transcripts of a second group of genes, designated Group II and comprising *HvCesA4*, *HvCesA7*, and *HvCesA8*, were also found in approximately equal relative abundance in a wide range of tissues and tissue segments (Figs. 5 and 7–10). Within this group, the relative abundance of mRNAs increased from *HvCesA7* through *HvCesA4* to *HvCesA8*. Transcription patterns of the Group I and Group II *HvCesA* genes were dramatically different (Figs. 5 and 7–9).

The other two members of the barley *HvCesA* gene family did not follow Group I or Group II transcription patterns and, therefore, appeared to be independently regulated. Levels of *HvCesA5* mRNA were extremely low in all tissues examined. However, *HvCesA3* mRNAs were more abundant, particularly toward the tips of young and mature leaves, where *HvCesA3* was the most abundant *CesA* mRNA by far (Fig. 5). Thus, *HvCesA3* may play a specific or specialized role in cellulose synthesis in these regions of barley leaves or may be involved in the synthesis of wall polysaccharides other than cellulose.

It can be concluded from these data that Q-PCR is sufficiently sensitive and precise to confidently detect co-expression of groups of genes through constant relative proportions of their mRNAs (Figs. 5 and 7–10), provided that transcript levels are carefully normalized (Fig. 4; Vandesompele et al., 2002). The fact that transcript levels within the barley

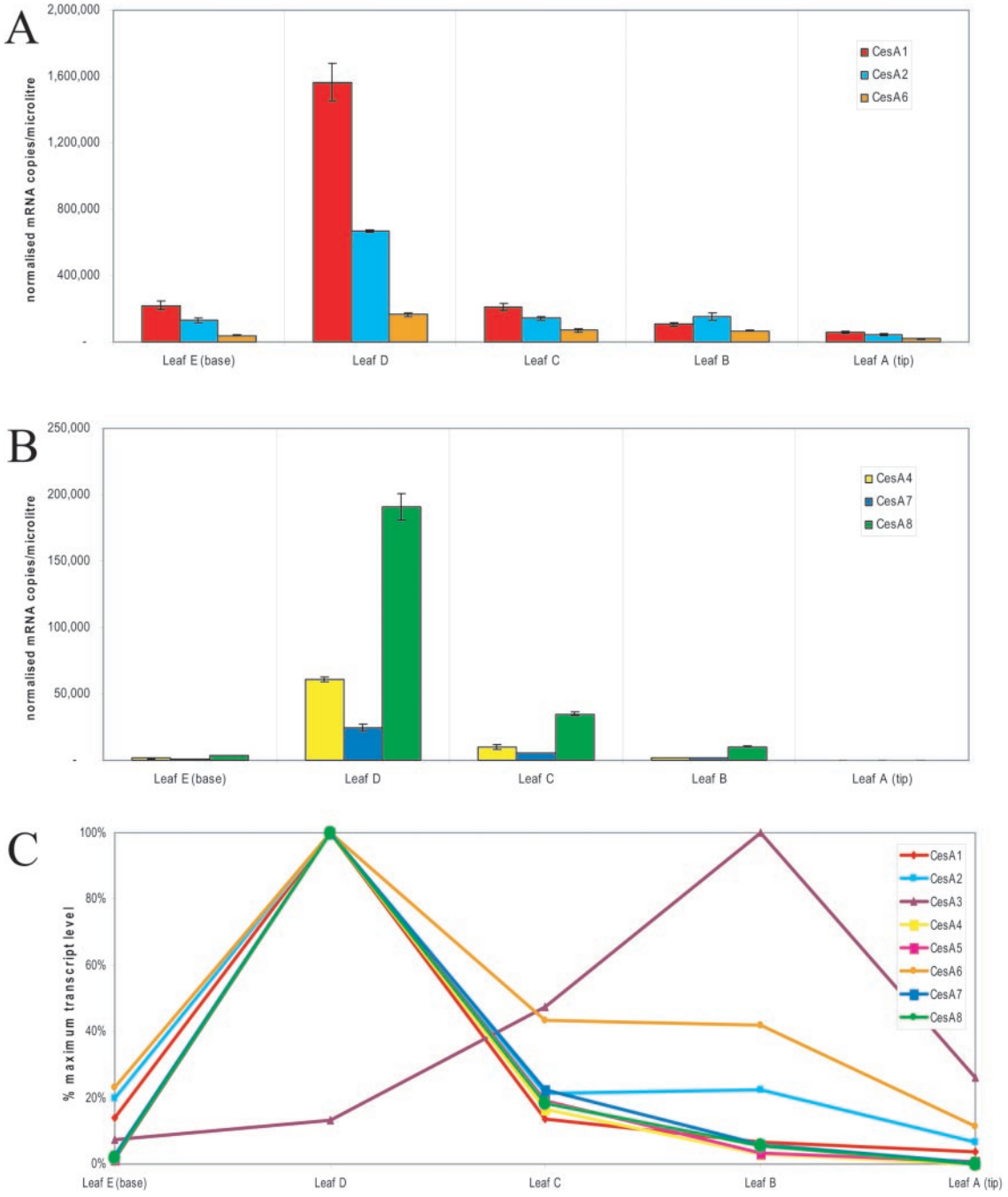

Figure 7. Normalized expression levels for the barley *HvCesA* genes in segments of young leaves. A, Levels of the Group I transcripts, *HvCesA1*, *HvCesA2*, and *HvCesA6*. B, Levels of the Group II transcripts, *HvCesA4*, *HvCesA7*, and *HvCesA8*. C, Levels of the eight *HvCesA* transcripts in segments of the young leaf (Fig. 6), expressed as a percentage of maximum transcript levels of each gene. The similar transcript profile for the Groups I and II genes can be seen, but *HvCesA3* transcripts are clearly distributed in a different pattern, with the highest abundance of mRNA just below the leaf tip in segment B. Error bars = SDs for each mRNA.

Group I or Group II genes are in approximately constant ratios, but not exactly the same, presumably relates to differences in stability and translational efficiencies of the individual mRNAs within the groups.

The co-expression patterns revealed by the Q-PCR in a wide range of barley tissues are suggestive of functional links between individual *HvCesA* genes. The three Group I *HvCesA1*, *HvCesA2*, and *HvCesA6* transcripts are most abundant in tissues and tissue

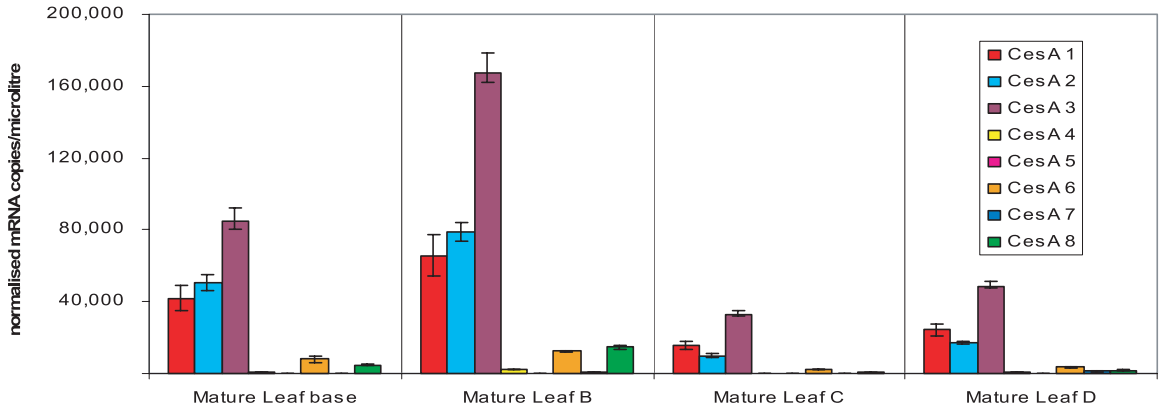

Figure 8. Normalized expression levels for the barley *HvCesA* genes in segments of mature leaves. In this case, *HvCesA3* transcripts are the most abundant, particularly in the middle region of the leaf. Error bars = SDs for each mRNA.

segments where primary cell wall synthesis would be expected to predominate (Figs. 5 and 7–9). It is possible that the protein products of the three genes are required for the formation of a single cellulose-synthesizing complex in rosettes of the plasma membrane during cell wall synthesis. In the model for rosette structure presented by Doblin et al. (2002), the rosettes could be constituted with different numbers of individual cellulose synthase isoforms; this is another possible explanation for the different levels of the three Group I *HvCesA1*, *HvCesA2*, and *HvCesA6* transcripts.

Similarly, the three Group II *HvCesA4*, *HvCesA7*, and *HvCesA8* genes appear to be co-expressed in tissues in which secondary wall synthesis would be proceeding, including stems and the maturation zones of roots (Fig. 5A). Consistent with this suggestion, Taylor et al. (2003) and Gardiner et al. (2003) have shown that three distinct CesA proteins, namely AtCesA4 (IRX5), AtCesA7 (IRX3), and AtCesA8 (IRX1), are jointly required for correct assembly of a cellulose synthesis complex in the secondary walls of Arabidopsis. Less direct but mounting evidence suggests that the three Arabidopsis AtCesA1, AtCesA3, and AtCesA6 proteins comprise a similar cellulose synthase complex that is required for primary cell wall biosynthesis (Arioli et al., 1998; Scheible et al., 2001; Desprez et al., 2002; Eckardt, 2003).

Williamson et al. (2002) have pointed out that much of the evidence for the role of multiple CesA proteins in the formation of functional cellulose synthase complexes has been based on genetic and inhibitor data and that major quantitative changes in expression levels of *CesA* genes implicated in primary and secondary wall synthesis need to be demonstrated to support the evidence from Arabidopsis mutants. The marked quantitative differences in Group I and Group II gene expression levels in different tissues and tissue segments of barley (Figs. 5 and 7–10) provide at least some evidence to support the genetic evidence from Arabidopsis. Furthermore, the barley *CesA* gene expression patterns add further, independent support for the suggestion that three different CesA proteins are required for the formation of cellulose-synthesizing complexes and that two such groups of three different proteins are required for cellulose synthesis in different tissues or at different developmental stages.

In contrast, semiquantitative RT-PCR of *ZmCesA* mRNA levels in maize and *OsCesA* mRNAs in rice did not reveal any obvious groups of genes with similar relative concentrations in different tissues (Holland et al., 2000; Vergara and Carpita, 2001). However, quantitative analyses of *ZmCesA* transcript levels through massively parallel signature sequencing indicated that the *ZmCesA1*, *ZmCesA2*, *ZmCesA7*, and *ZmCesA8* genes could be coordinately expressed (Dhugga, 2001). In rice, the three genes implicated as causative agents for three brittle culm mutations are *OsCesA4*, *OsCesA7*, and *OsCesA9*; these genes are thought to be required for the formation of a functional CesA unit for secondary wall cellulose synthesis (Tanaka et al., 2003).

The emerging evidence that groups of three different CesA proteins might be required for cellulose biosynthesis, in both primary and secondary walls, and in both monocotyledons and dicotyledons (Figs. 5 and 7–10; Eckardt, 2003), raises a fundamental question about orthologous members of multigene families in plants. More specifically, do corresponding orthologous members from Arabidopsis and barley *CesA* gene families perform the same functions in the two relatively divergent species, and can one predict the functions of individual members of a gene family based on known functions of orthologous genes from another species? Examination of the phylogenetic tree (Fig. 3; Table I) suggests that the answer to the first question is positive, at least in the case of the *CesA* gene families. The amino acid sequences deduced from the three Group I barley *HvCesA* genes, *HvCesA1*, *HvCesA2*, and *HvCesA6*, are most closely related to the Arabidopsis *AtCesA3*, *AtCesA5*, and *AtCesA1* genes, respectively, although it should be noted that *AtCesA5* is very similar to

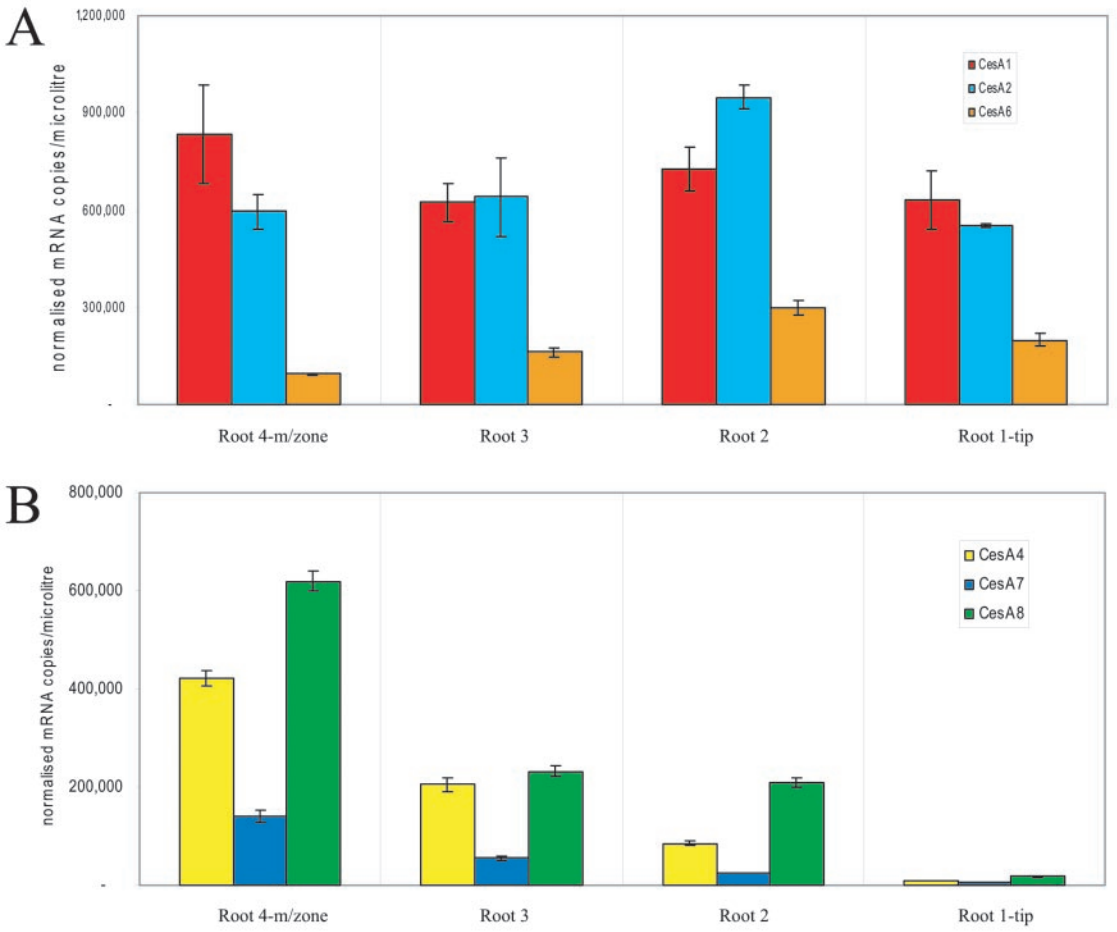

Figure 9. Normalized expression levels for the barley *HvCesA* genes in segments of young roots. A, Levels of the Group I transcripts, *HvCesA1*, *HvCesA2*, and *HvCesA6*. B, Levels of the Group II transcripts, *HvCesA4*, *HvCesA7*, and *HvCesA8*. In this case, *HvCesA3* transcripts showed a distribution similar to that for the Group II mRNAs (data not shown). The positions of the root segments are shown in Figure 6. Error bars = SDs for each mRNA.

AtCesA2, *AtCesA6*, and *AtCesA9*, all of which are located together in one branch of the phylogenetic tree (Fig. 3; Table I). Current genetic evidence indicates that Arabidopsis *AtCesA3*, *AtCesA6*, and *AtCesA1* genes, which are orthologous to the barley Group I genes, are required for cellulose synthase complex formation during primary wall synthesis (Figs. 5 and 7–10; Eckardt, 2003). Similarly, the three Group II barley *HvCesA4*, *HvCesA7*, and *HvCesA8* genes are most similar to the Arabidopsis *AtCesA4*, *AtCesA8*, and *AtCesA7* genes, respectively (Fig. 3; Table I). This group of three Arabidopsis genes has been implicated in secondary cell wall synthesis (Figs. 5 and 7–10; Gardiner et al., 2003; Taylor et al., 2003). Thus, it can be concluded that the two groups of three CesA proteins in Arabidopsis that have been linked genetically to primary and secondary wall cellulose deposition are orthologous to the Group I and Group II co-expressed barley genes, respectively.

Despite these similarities, it must be clearly stated at this stage that the roles of the barley Group I and Group II CesA proteins in primary and/or secondary wall synthesis remain to be demonstrated unequivocally. We have tried tissue printing and in situ PCR procedures in attempts to correlate Group I and Group II transcripts with primary and secondary wall formation (data not shown) but have been unable to convincingly show that specific transcripts are more abundant in cells synthesizing the two different wall types. It is likely that higher resolution immunogold labeling of specific HvCesA proteins will be necessary to define the precise cellular location of individual cellulose synthases in various tissues at different stages of growth and development. In the absence of well-characterized barley mutant libraries, it also will be necessary to analyze the functions of individual *HvCesA* genes through specific gene silencing, both transiently and in stably transformed plants.

MATERIALS AND METHODS

Plant Material

Barley (*Hordeum vulgare* L. cv Sloop) plants were grown in a greenhouse under a day/night temperature regime of 23°C/15°C. For non-greenhouse-

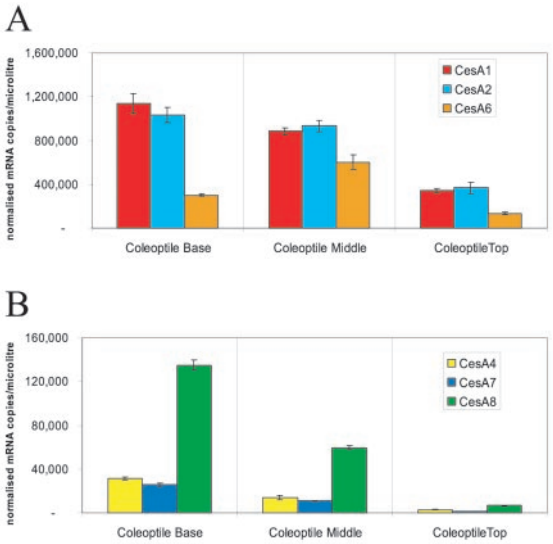

Figure 10. Normalized expression levels for the barley *HvCesA* genes in segments of coleoptiles. A, Levels of the Group I transcripts, *HvCesA1*, *HvCesA2*, and *HvCesA6*. B, Levels of the Group II transcripts, *HvCesA4*, *HvCesA7*, and *HvCesA8*. In this case, *HvCesA3* transcripts remained at similar levels in each segment and, therefore, were different to the distribution of both the Groups I and II mRNAs (data not shown). Positions of the coleoptile segments are shown in Figure 6. Error bars = SDs for each mRNA.

grown samples, grain was germinated either in damp vermiculite or on damp paper towels in the dark for 3 to 6 d at 20°C. At harvest, seedling leaves from vermiculite grown grain were about 75% of their final length. Seedling leaves of about 13 cm in length were used to isolate leaf tip (the top 7 mm of the leaf) and 3 mm at the base (cell division zone; Fig. 5). In addition, seedling leaf blades were divided into five sections, designated sections A (leaf tip) to E (leaf base; Fig. 6). Segment E would contain dividing and some elongating cells; at segment D elongation would be complete and secondary wall synthesis would be under way; and in segments C and B, there would be no growth but wall maturation would be occurring (Schünmann et al., 1997; Wenzel et al., 2000). For mature leaves, 1-cm sections were excised from the fifth leaf when it was 32 cm long and about 75% of its final length; the segments were 8, 16, 24, and 29 cm from the base of the leaf.

Similarly, root tip (1 cm, containing root cap, meristem, and elongation zone) and mature root (1-cm section about 6 cm behind the root tip, containing the differentiation and maturation zone) were harvested (Fig. 5). Selected 1-cm sections of roots harvested after 5 d were also excised (Fig. 6). In the root tip, cell elongation would be occurring; in segment 2, growth would be complete, but secondary wall synthesis would begin; in segment 3, the xylem would be maturing, whereas in segment 4, lateral root formation would be occurring (Fig. 6; B. Atwell, personal communication).

Three-day coleoptiles were divided into three sections of about 1 cm each (designated tip, middle, and base; Fig. 6). Cells in the basal region were expanding, those in the middle segment were fully expanded, and those in the tip were generally shorter; secondary wall formation would be restricted to the two small vascular bundles. Floral tissues, consisting of anthers and pistil, were collected about 2 weeks before anthesis and at anthesis. Stem tissue was taken from the upper internode, below the pre-anthesis spike (i.e. below the peduncle); cell elongation would have ceased in this segment. Developing grain was collected 3 and 13 DPA.

Cloning *HvCesA* cDNAs and Genes

A barley cDNA library derived from RNA of suspension-cultured cell lines was prepared in λ-ZAPII, according to the manufacturer's instructions (Stratagene, La Jolla, CA) and screened with a PCR fragment amplified with degenerate primers designed to match conserved regions in plant *CesA* genes. Subsequently, unique 3′-UTR probes were generated by PCR and used to screen the barley BAC library (cv Morex) obtained from the Clemson University Genomics Institute (SC; http://www.genome.clemson.edu). DNA from each positive BAC was isolated and used in a modified genomic walking procedure (Siebert et al., 1995) with restriction enzymes *Dra*I, *Sma*I, *Stu*I, *Eco*RV, *Hinc*II, *Sca*I, *Nru*I, *Pml*I, *Pvu*II, and *Sna*BI. Additional genomic sequence for some of the *HvCesA* genes was obtained in this manner.

Full or partial cDNAs in one contiguous segment were obtained by PCR using the Elongase *Taq* polymerase (Invitrogen, Carlsbad, CA) and primers designed to the 5′- and 3′-UTRs of the *HvCesA* cDNAs, using various cDNA populations prepared from the tissues listed above as templates. The cDNAs were cloned into the pGEM-T Easy vector (Promega, Madison, WI), and both strands were sequenced using Big Dye 3 chemistry on an ABI 3700 (Applied Biosystems, Foster City, CA) capillary sequencer.

Mapping the *HvCesA* Genes

The barley doubled haploid mapping populations Chebec × Harrington (120 lines), Galleon × Haruna Nijo (112 lines), and Clipper × Sahara (150 lines) were used to map the *HvCesA* genes (Karakousis et al., 2003). Peter Langridge (University of Adelaide, Australia) kindly supplied filters of digested genomic DNA for Southern hybridization analyses. Loci were positioned using the "find best location" function of Map Manager QT version b29ppc (Manly and Olson, 1999). Where polymorphisms could not be detected, the genes were mapped to the chromosome level, using a series of wheat barley addition lines (Islam et al., 1981).

Alignments and Analyses of EST Databases

Multiple sequence alignments and an unrooted, radial phylogenetic tree of the barley *HvCesA* gene family were generated using ClustalX (Thompson et al., 1997). The sequences of approximately 314,000 barley ESTs available through the public databases (http://www.ncbi.nlm.nih.gov) were searched for barley *HvCesA* sequences, and, to estimate the number of genes in the *HvCesA* family, *HvCesA* sequences listed on the Stanford University site (http://cellwall.stanford.edu/; Richmond and Somerville, 2000) were subsequently aligned with the cloned cDNA sequences from the present study. Nonoverlapping EST sequences that corresponded to fragments of the same gene thereby could be identified.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from at least three individual samples of all tissues, using a commercially prepared guanidine reagent, TRIzol (Invitrogen) according to the manufacturer's instructions. Purified RNA was treated with DNaseI using the DNA-free kit (Ambion, Austin, TX) according to the manufacturer's instructions. RNA integrity was checked on a 1.6% (w/v) agarose gel containing ethidium bromide.

For cDNA synthesis, four independent reactions were undertaken for each tissue and the products pooled. Thus, 2 μg of each RNA was mixed with 1 μL of 50 nM oligo-dT(12-20) primer, 1 μL of 10 mM dNTP mix, and sterile water to a volume of 10 μL. The reaction was heated at 65°C for 5 min and snap cooled on ice. A master mix (10 μL) was added to each reaction and contained 2× RT buffer, 0.1 M dithiothreitol, 50 mM $MgCl_2$, 40 units of RNAseOUT (Invitrogen), and 50 units of Superscript II RT. The reaction was incubated at 42°C for 1 h and for 15 min at 75°C. The cDNA was stored at −20°C.

Q-PCR Analysis of Transcript Concentrations

The primer pairs for control genes and specific *HvCesA* genes were designed for barley var. Sloop and are listed in Table III. Stock solutions of the PCR product were prepared from a cDNA population generated from 3-d-old coleoptile RNA, purified, and quantified by HPLC. The coleoptile-derived cDNA (1 μL) was amplified in a reaction containing 10 μL of QuantiTect SYBR Green PCR reagent (Qiagen, Valencia, CA), 3 μL each of 4 μM forward and reverse primers, and 3 μL of water. The amplification was effected in a RG 2000 Rotor-Gene Real Time Thermal Cycler (Corbett Research, Sydney) as follows: 15 min at 95°C followed by 45 cycles of 20 s at 95°C, 30 s at 55°C, 30 s at 72°C, and 15 s at 80°C. A melt curve was obtained

from the PCR product at the end of the amplification by heating from 70°C to 99°C. During the amplification, fluorescence data was acquired at 72°C and 80°C to gauge the abundance of the individual genes in the coleoptile cDNA preparation. From the melt curve, the optimal temperature for data acquisition was determined (Table III).

Between four and six independent 20 μL PCR reaction mixes were combined and purified by HPLC (Wong et al., 2000) on an Agilent Eclipse DS DNA 2.1-mm × 15-cm 3.5-micron reverse phase column (Agilent Technologies, Palo Alto, CA). Chromatography was performed using buffer A (100 mM triethylammonium acetate and 0.1 mM EDTA) and buffer B (100 mM triethylammonium acetate, 0.1 mM EDTA, and 25% [w/v] acetonitrile). The gradient was applied at a flow rate of 0.2 mL min^{-1} at 40°C, as follows: 0 to 30 min with 35% (w/v) buffer B, 30 to 31 min with 70% (w/v) buffer B, 31 to 40 min with 35% (w/v) buffer B, and after 40 min, 35% (w/v) buffer B. The DNA was detected by A_{260}. The purified PCR products were quantified by comparison of the peak area with the areas of three of the peaks in a pUC19/*Hpa*II digest (Geneworks, Adelaide, Australia). In 2 μL of a 500 ng μL^{-1} digest, the peaks used for reference were 147 bp, representing 55 ng, 190 bp (71 ng), and 242 bp (90 ng). From these data, an average value for nanograms per unit area of a peak was calculated. This value was used to determine the mass of the purified PCR product. The value was determined with every batch of PCR products purified. The product was dried and dissolved in water to produce a 20 ng μL^{-1} stock solution. The size in base pairs and identity of PCR products was confirmed by sequencing. An aliquot of this solution was diluted to produce a stock solution containing 10^9 copies of the PCR product per microliter.

A dilution series covering seven orders of magnitude was prepared from the 10^9 copies μL^{-1} stock solution to produce solutions covering 10^7 to 10^1 μL^{-1}. Three replicates of each of the seven standard concentrations were included with every Q-PCR experiment, together with a minimum of two "no template" controls. For all genes except *HvCesA5*, a 1:10 dilution of the cDNA was sufficient to produce expression data with an acceptable SD. Undiluted cDNA was required for the determination of *HvCesA5* expression levels because of its low abundance. Four replicate PCRs for each of the cDNAs were included in each experiment.

For the Q-PCR experiments, 1 μL cDNA solution was used in a reaction containing 10 μL of QuantiTect SYBR Green PCR reagent, 3 μL each of the forward and reverse primers at 4 μM, 0.6 μL 10× SYBR Green in water (freshly diluted 10,000× in dimethyl sulfoxide), and 2.4 μL of water. Reactions were performed as follows: 15 min at 95°C followed by 45 cycles of 20 s at 95°C, 30 s at 55°C, 30 s at 72°C, and 15 s at the optimal acquisition temperature (Table III). A melt curve was obtained from the product at the end of the amplification by heating from 70°C to 99°C. PCR products were separated by electrophoresis in 2.5% (w/v) agarose-Tris-borate/EDTA-ethidium bromide gels. The Rotor-Gene V4.6 software (Corbett Research) was used to determine the optimal cycle threshold from the dilution series, and the mean expression level and SDs for each set of four replicates for each cDNA were calculated.

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LITERATURE CITED

Arioli T, Peng L, Betzner AS, Burn J, Wittke W, Herth W, Camilleri C, Hofte H, Plazinski J, Birch R et al. (1998) Molecular analysis of cellulose biosynthesis in *Arabidopsis*. Science **279:** 717–720

Beeckman T, Przemeck GHK, Stamatiou G, Lau R, Terryn N, de Rycke R, Inze D, Berleth T (2002) Genetic complexity of cellulose synthase A gene function in *Arabidopsis* embryogenesis. Plant Physiol **130:** 1883–1893

Burn JE, Hocart CH, Birch RJ, Cork AC, Williamson RE (2002) Functional analysis of the cellulose synthase genes *CesA1,* CesA2, and CesA3 in Arabidopsis. Plant Physiol **129:** 797–807

Burton RA, Gibeaut DM, Bacic A, Findlay K, Roberts K, Hamilton A, Baulcombe DC, Fincher GB (2000) Virus-induced silencing of a plant cellulose synthase gene. Plant Cell **12:** 691–705

Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol **25:** 169–193

Caño-Delgado A, Penfield S, Smith C, Catley M, Bevan M (2003) Reduced cellulose synthesis invokes lignification and defense responses in *Arabidopsis thaliana*. Plant J **34:** 351–362

Carpita NC (1996) Structure and biogenesis of the cell walls of grasses. Annu Rev Plant Physiol Plant Mol Biol **47:** 445–476

Delmer DP (1999) Cellulose synthesis: exciting times for a difficult field. Annu Rev Plant Physiol Plant Mol Biol **50:** 245–276

Desprez T, Vernhettes S, Fagard M, Refregier G, Desnos T, Aletti E, Py N, Pelletier S, Hofte H (2002) Resistance against herbicide isoxaben and cellulose deficiency caused by distinct mutations in same cellulose synthase isoform CESA6. Plant Physiol **128:** 482–490

Dhugga KS (2001) Building the wall: genes and enzyme complexes for polysaccharide synthases. Curr Opin Plant Biol **4:** 488–493

Doblin MS, Kurek I, Jacob-Wilk D, Delmer DP (2002) Cellulose biosynthesis in plants: from genes to rosettes. Plant Cell Physiol **43:** 1407–1420

Eckardt NA (2003) Cellulose synthesis takes the CesA train. Plant Cell **15:** 1685–1688

Fagard M, Desnos T, Desprez T, Goubet F, Refregier G, Mouille G, McCann M, Rayon C, Vernhettes S, Hofte H (2000) PROCUSTE1 encodes a cellulose synthase required for normal cell elongation specifically in roots and dark-grown hypocotyls of *Arabidopsis*. Plant Cell **12:**2409–2423

Fincher GB, Stone BA (1993) The physiology and biochemistry of germination in barley. *In* AW MacGregor, R Bhatty, S Barley, eds, Chemistry and Technology. American Association of Cereal Chemists, St. Paul, MN pp 247–295

Fink L, Seeger W, Ermert L, Hanze J, Stahl U, Grimminger F, Kummer W, Bohle RM (1998) Real-time quantitative RT-PCR after laser-assisted cell picking. Nat Med **4:** 1329–1333

Gardiner JC, Taylor NG, Turner SR (2003) Control of cellulose synthase complex localization in developing xylem. Plant Cell **15:** 1740–1748

Haendler B, Hofer-Warbinek R, Hofer E (1987) Complementary DNA for human T-cell cyclophilin. EMBO J **6:** 947–950

Holland N, Holland D, Helentjaris T, Dhugga KS, Xoconostle-Cazares B, Delmer DP (2000) A comparative analysis of the plant cellulose synthase (*CesA*) gene family. Plant Physiol **123:** 1313–1323

Islam AKMB, Shepherd KW, Sparrow DHB (1981) Isolation and characterization of euplasmic wheat-barley addition lines. Heredity **46:** 161–174

Karakousis A, Gustafson JP, Chalmers KJ, Barr AR, Langridge P (2003) A consensus map of barley integrating SSR, RFLP and AFLP markers. Aust J Ag Res (in press)

Kimura S, Laosinchai W, Itoh T, Cui X, Linder CR, Brown RM Jr (1999) Immunogold labeling of rosette terminal cellulose-synthesizing complexes in the vascular plant *Vigna angularis*. Plant Cell **11:** 2075–2085

Lai-Kee-Him J, Chanzy H, Muller M, Putaux JL, Imai T, Bulone V (2002) In vitro versus in vivo cellulose microfibrils from plant primary wall synthases: structural differences. J Biol Chem **277:** 36931–36939

Manly KF, Olson JM (1999) Overview of QTL mapping software and introduction to Map Manager QT. Mammalian Genome **10:** 327–334

Mueller SC, Brown RM Jr (1980) Evidence for an intramembranous component associated with a cellulose microfibril synthesizing complex in higher plants. J Cell Biol **84:** 315–326

Ozturk ZN, Talame V, Deyholos M, Michalowski CB, Galbraith DW, Gozukirmizi N, Tuberosa R, Bohnert HJ (2002) Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. Plant Mol Biol **48:** 551–573

Pear JR, Kawagoe Y, Schreckengost WE, Delmer DP, Stalker DM (1996) Higher plants contain homologs of the bacterial celA genes encoding the catalytic subunit of cellulose synthase. Proc Natl Acad Sci USA **93:** 12637–12642

Richmond TA, Somerville CR (2000) The cellulose synthase superfamily. Plant Physiol **124:** 495–498

Roelofsen A (1958) Cell wall structure as related to surface growth. Acta Bot Neerl **7:** 77–89

Scheible W-R, Eshed R, Richmond T, Delmer D, Somerville C (2001) Modifications of cellulose synthase confer resistance to isoxaben and thiazolidinone herbicides in *Arabidopsis ixr1* mutants. Proc Natl Acad Sci USA **98:** 10079–10084

Schünmann PHD, Smith RC, Lång V, Matthews PR, Chandler PM (1997) Expression of XET-related genes and its relation to elongation in leaves of barley (*Hordeum vulgare* L.). Plant Cell Environ **20:** 1439–1450

Siebert PD, Chenchik A, Kellog DE, Lukyanov KA, Lukyanov SA (1995) An improved PCR method for walking in uncloned genomic DNA. Nucleic Acids Res **23:** 1087–1088

Tanaka K, Murata K, Yamazaki M, Onosato K, Miyao A, Hirochika H (2003) Three distinct rice cellulose synthase catalytic subunit genes required for cellulose synthesis in the secondary wall. Plant Physiol **133:** 73–83

Taylor NG, Howells RM, Huttly AK, Vickers K, Turner SR (2003) Interactions among three distinct CesA proteins essential for cellulose synthesis. Proc Natl Acad Sci USA **100:** 1450–1455

Taylor NG, Laurie S, Turner SR (2000) Multiple cellulose synthesis catalytic subunits are required for cellulose synthesis in *Arabidopsis*. Plant Cell **12:** 2529–2539

Taylor NG, Scheible WR, Cutler S, Somerville CR, Turner SR (1999) The irregular *xylem3* locus of *Arabidopsis* encodes a cellulose synthase required for secondary cell wall synthesis. Plant Cell **11:** 769–780

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res **25:** 4876–4882

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol **3:** 1–11

Vergara CE, Carpita NC (2001) β-D-Glycan synthases and the *CesA* gene family: lessons to be learned from the mixed-linkage (1→3),(1→4)β-d-glucan synthase. Plant Mol Biol **47:** 145–160

Wenzel CL, Williamson RE, Wasteneys GO (2000) Gibberellin-induced changes in growth anisotropy precede gibberellin-dependent changes in cortical microtubule orientation in developing epidermal cells of barley leaves: kinematic and cytological studies of a gibberellin-responsive dwarf mutant, M489. Plant Physiol **124:** 813–822

Williamson RE, Burn JE, Hocart CH (2002) Towards the mechanism of cellulose synthesis. Trends Plant Sci **7:** 461–467

Wong LY, Belonogoff V, Boyd VL, Hunkapiller NM, Casey PM, Liew S-N, Lazaruk KD, Baumhueter S (2000) General method for HPLC purification and sequencing of selected dsDNA gene fragments from complex PCRs generated during gene expression profiling. BioTechniques **28:** 776–783